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EFFECT OF ADRENALECTOMY ON CADMIUM- AND TURPENTINE-INDUCED HEPATIC SYNTHESIS OF METALLOTHIONEIN AND α_2-MACROFETOPROTEIN IN THE RAT[1,2]

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Abstract—Recent reports have strongly implicated glucocorticoids in the induction of hepatic metallothionein synthesis and hypozincemia which occurs in certain pathophysiologic conditions. Studies were performed in rats to determine the effect of adrenalectomy and glucocorticoid treatment on the hepatic accumulation of metallothionein subsequent to the administration of cadmium and turpentine, two diverse substances known to induce hypozincemia and hepatic synthesis of metallothionein as well as α_2-macrofetoprotein in intact rats. By 24 h, both substances induced significant hypozincemia, hepatic metallothionein accumulation, and a severe tissue inflammatory response in adrenalectomized rats. Adrenalectomy only prevented the increase in plasma α_2-macrofetoprotein concentration. Results indicate that hepatic synthesis of α_2-macrofetoprotein, but not metallothionein, is mediated by adrenal hormones. Thus, glucocorticoids do not play a "vital" role in hepatic metallothionein accumulation or hypozincemia induced by inflammatory stress, as previously postulated.

INTRODUCTION

There is considerable interest in the mechanism(s) involved in the induction of hepatic metallothionein synthesis, since this pleomorphic low-molecular-weight metalloprotein (1) appears to have a key role in zinc metabolism in

[1]In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[2]The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense (Para. 4-3, AR 360-5).

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normal (2) as well as in several pathophysiologic (3–5) and stress conditions (6) characterized by hypozincemia and sequestration of endogenous Zn in the liver. Hepatic synthesis of this heavy-metal-binding protein has been shown to be regulated at translational and transcriptional levels (7, 8).

Recent reports have suggested that induction of hepatic metallothionein synthesis during various stress conditions may be regulated in part by adrenal steroids (9, 10). This concept is seemingly supported by evidence indicating that certain glucocorticoids mediate the synthesis of metallothionein in rat hepatocytes (9, 11) and HeLa cells in culture (12). In addition, administration of dexamethasone, a potent synthetic glucocorticoid analog, to adrenalectomized rats has been reported to induce hypozincemia and hepatic synthesis of metallothionein (10). Further, it is well known that glucocorticoids affect the synthesis of specific mRNA (13) and mediate the de novo hepatic synthesis of certain acute-phase proteins (14). However, dexamethasone has also been shown to increase the hepatic uptake of Zn in rat liver parenchymal cells (15). Thus, it is unclear whether the observed influence of glucocorticoid (dexamethasone) on hepatic metallothionein synthesis reflects hormone-mediated gene expression or cellular accumulation of zinc (10) or both, since Zn and dexamethasone may be "primary inducers" of metallothionein (9).

Other evidence presented by Flynn et al. (16) indicates that the pituitary–adrenal axis may be important in maintenance of circulating Zn and mobilization of body Zn stores. However, we have presented some preliminary evidence (17) obtained with hypophysectomized rats which suggests that hypozincemia and hepatic metallothionein induction by diverse stimuli such as Cd, turpentine, and isoproterenol is not dependent on an intact adrenal–pituitary axis. Studies were therefore performed to delineate further the potential role of the adrenal in mediating hypozincemia and the hepatic synthesis of metallothionein after the administration of Cd and turpentine to adrenalectomized and intact rats. Since these substances induce inflammation (17), we concurrently followed the appearance of plasma α_2-macrofetoprotein (α_2MFP), an acute-phase protein in the rat, synthesized by the liver in response to inflammatory stress and whose synthesis has been shown to be influenced by glucocorticoids (14).

MATERIALS AND METHODS

Intact male Fisher-Dunning rats weighing 236 ± 9 g (mean ± *SEM*) and commercially obtained adrenalectomized rats weighing 279 ± 11 g were acclimated for a minimum of one week under controlled environmental conditions (3) prior to use in experiments. Food and water were provided ad libitum, except that physiological saline rather than regular tap water was provided to adrenalectomized (ADREX) rats. Food was withheld from all rats after the administration of Cd and turpentine, as described below.

Hypozincemia and hepatic metallothionein accumulation were induced by the subcutaneous administration of $CdCl_2$ dissolved in physiological saline (0.6 mg/100 g body weight) or turpentine (1.0 ml/rat). Control rats received an equivalent volume of saline. In certain experiments, ADREX rats were pretreated with a single intramuscular injection of hydrocortisone sodium succinate (0.15 mg/100 g) on four consecutive days with Cd or turpentine administered on day 4. Additionally, other ADREX rats were treated with a single intraperitoneal injection of dexamethasone (disodium potassium salt, 0.2 mg/100 g) immediately after Cd administration. All rats were killed by exsanguination 24 h after Cd or turpentine injection. Heparinized blood samples were obtained from the pleural cavity after transection of the vena cava, plasma separated by centrifugation for subsequent determination of Zn and Cd by atomic absorption spectrophotometry (3), and α_2MFP by an automated immunoprecipitin method employing goat anti-rat α_2MFP (18). Livers were extirpated within 1 min following exsanguination and processed for the isolation and measurement of metallothionein–Zn and –Cd concentrations by the methods described by Sobocinski et al. (3). The identity of isolated metallothionein forms 1 and 2 was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (3).

Tissues were obtained for histologic examination from injection sites selected at random from rats administered the various substances. Tissue samples were stretched and mounted on cardboard and immediately placed in 10% neutral buffered formalin and processed as previously described (5).

Significance of differences between group means was determined by analysis of variance with a $P < 0.01$ considered significant.

RESULTS

The effect of Cd and turpentine administration on plasma Zn and α_2MFP concentrations in intact, ADREX, and hydrocortisone-supplemented ADREX rats is shown, together with the respective metallothionein–Zn and –Cd concentrations, in Table 1. Cadmium and turpentine produced significant hypozincemia, as well as increases in plasma α_2MFP and hepatic metallothionein concentrations in intact rats compared to saline-injected controls. Although adrenalectomy effectively abolished the increases in α_2MFP, it did not prevent either the plasma Zn depression or hepatic metallothionein accumulation induced by Cd and turpentine. On a mole basis, the mean ratio of Cd–Zn bound to metallothionein was calculated to be 1.0 ± 0.1 (*SEM*) in Cd-treated rats. Of the two metals measured, zinc was the major metal bound to metallothionein in turpentine-treated rats. The concentration of total metal bound in turpentine-treated rats never exceeded that found in Cd-treated rats. Hydrocortisone effectively promoted the appearance of α_2MFP in the plasma of ADREX rats administered turpentine. However, hydrocortisone supplementation did not fully restore the plasma α_2MFP concentration in ADREX rats administered Cd to that observed in intact rats. Hydrocortisone administration to ADREX control rats did not significantly alter any of the measured parameters.

In marked contrast with the lack of a significant effect of hydrocortisone on the plasma α_2MFP concentration in Cd-treated ADREX rats, the

Table 1. Effect of Cd and Turpentine Administration on Plasma Zn, α_2-Macrofetoprotein (α_2MFP) and Hepatic Metallothionein–Zn and –Cd Concentration in Intact, Adrenalectomized (ADREX), and Hydrocortisone (HC) Supplemented ADREX Rats

		Mean ± *SEM* (*N*)			
		Plasma		Metallothionein (μg/g liver, wet weight)	
Group	Treatment[a]	Zn (μg/100 ml)	α_2MFP[b] (%)	*Zn*	*Cd*
ADREX	*Cd*	56.3 ± 6.3[c] (5)	5.8 ± 0.3[d] (10)	15.9 ± 0.9[c] (10)	26.8 ± 1.7[c] (10)
	Turpentine	73.1 ± 6.2[c] (10)	8.6 ± 0.5[d] (10)	6.4 ± 0.3[c] (10)	< 0.3
	Saline	132.5 ± 4.1 (5)	7.1 ± 0.6 (5)	1.4 ± 0.4 (5)	< 0.3
Intact	Cd	47.4 ± 5.2[c] (8)	23.9 ± 2.0[c] (10)	11.2 ± 0.7[c] (9)	22.1 ± 1.3[c] (9)
	Turpentine	91.9 ± 4.1[c] (7)	33.1 ± 7.0[c] (10)	8.5 ± 0.8[c] (9)	< 0.3
	Saline	127.3 ± 3.9 (13)	6.6 ± 0.6 (13)	1.8 ± 0.3 (13)	< 0.3
ADREX + HC	*Cd*	57.5 ± 3.1[c] (5)	10.8 ± 1.5 (5)	13.0 ± 0.9[c] (5)	23.7 ± 1.2[c] (5)
	Turpentine	81.0 ± 4.8[c] (5)	37.0 ± 7.1[c] (5)	7.3 ± 0.9[c] (5)	< 0.3
	Saline	121.1 ± 5.0 (5)	7.6 ± 0.5 (5)	0.8 ± 0.2 (5)	< 0.3

[a]Rats were injected subcutaneously with $CdCl_2$ (0.6 mg/100 g body weight) dissolved in physiological saline or turpentine (1.0 ml/rat) 24 h prior to sacrifice. Control rats received an equivalent volume of saline (1.0 ml). Hydrocortisone was injected intramuscularly as described in Materials and Methods.

[b]Value expressed as a percent of the α_2MFP content of a pooled reference plasma obtained 48 h after subcutaneous injection of turpentine.

[c]Significantly different ($P < 0.01$) compared to saline control.

[d]Significantly different ($P < 0.01$) compared to intact group administered the same treatment.

Table 2. Effect of Cd Administration on Plasma Zn, α_2-Macrofetoprotein (α_2MFP), and Hepatic Metallothionein-Zn and -Cd Concentration in Adrenalectomized (ADREX) Rats With and Without Dexamethasone (DEX) Supplementation

	Mean ± *SEM*			
	Plasma		Metallothionein (μg/g liver, wet weight)	
Treatment[a] (*N*)	Zn (μg/100 ml)	α_2MFP[b] (%)	Zn	Cd
DEX (7)	116.0 ± 6.4	11.3 ± 0.4[c]	5.3 ± 0.4	< 0.3
Saline (7)	124.0 ± 6.7	7.0 ± 0.4	1.7 ± 0.3	< 0.3
Cd + DEX (7)	61.4 ± 5.2[c]	57.4 ± 7.6[c]	16.5 ± 0.9[c]	22.5 ± 1.1[c]
Cd + Saline (7)	58.9 ± 4.6[c,d]	7.7 ± 0.4	14.6 ± 1.6[c,d]	25.1 ± 2.5[c,d]

[a]Rats were injected subcutaneously with $CdCl_2$ (0.6 mg/100 g body weight) dissolved in physiological saline 24 h prior to sacrifice. Control rats received an equivalent volume of saline (1.0 ml). Dexamethasone was injected intraperitoneally immediately after Cd administration at a dose of 0.2 mg/100 g body weight.
[b]Value expressed as a percent of the α_2MFP content of a pooled reference plasma obtained 48 h after subcutaneous injection of turpentine.
[c]Significantly different ($P < 0.01$) compared to saline control.
[d]Not significantly different ($P < 0.01$) compared to dexamethasone-treated rats administered Cd.

administration of dexamethasone, at a dose approximately 40 times greater than the hydrocortisone replacement dose, effectively restored the α_2MFP response (Table 2). In addition, dexamethasone treatment of ADREX rats produced a slight depression in plasma Zn concentration and an increase ($P < 0.05$) in hepatic metallothionein-Zn concentration compared to saline controls. These data concerning dexamethasone effects on plasma Zn and hepatic metallothionein concentrations are consistent with previously published observations (10). However, dexamethasone administration to ADREX rats treated with Cd did not significantly affect the Cd-induced hypozincemia or hepatic metallothionein accumulation. Dexamethasone alone induced a significant increase in plasma α_2MFP in ADREX rats compared to saline controls. This finding is in agreement with other observations that glucocorticoid enhances the α_2MFP response in surgically traumatized rats (21).

SDS-PAGE separation of the two forms of hepatic metallothionein isolated from Cd- and turpentine-treated intact rats was identical to that previously described for metallothionein isolated from livers of infected rats (3). Normally only small quantities of metallothionein were isolated from saline-treated controls.

Subcutaneous administration of turpentine as well as Cd produced inflammatory lesions which were readily apparent upon histologic examination of tissues obtained from injection sites. The prominent microscopic

Fig. 1. Turpentine-treated intact rat. Multiple subcutaneous abscesses (A) consisting of broad bands of polymorphonuclear cells. Patchy areas of necrosis, edema, and inflammatory cells extend into the surrounding tissue. ×20 (reduced 10% for reproduction).

feature of the injection sites in turpentine-treated rats was the formation of subcutaneous abscesses (Figure 1). The subcutis was severely distended by broad rims of intense suppuration that formed around what appeared to be pockets of residual turpentine. Peripheral to these abscesses, there was extensive tissue necrosis and edema that extended to the overlying dermis and the subjacent deep abdominal musculature. These areas were diffusely infiltrated by large numbers of predominantly polymorphonuclear cells and a few macrophages.

The subcutaneous injection sites of intact Cd-treated rats were markedly distended by edema and patchy, often linear, areas of coagulative necrosis and hemorrhage (Figure 2). Vessels within the lesion were severely dilated and congested (Figure 3). The endothelial cells of these vessels were often pyknotic; frequently small areas of hemorrhage surrounded the walls of necrotic vessels. In contrast to the turpentine lesions, there were fewer numbers of inflammatory cells in injection sites. Small aggregates of polymorphonuclear cells (many of which were pyknotic and karyorrhectic) were associated with necrotic fibers of the cutaneous trunci muscle (Figure 4).

Fig. 2. Cd-treated intact rat. *The dermis and subcutis are distended by severe fibrinous* edema and patchy areas of coagulative necrosis. ×20 (reduced 5% for reproduction).

The lesions in the hydrocortisone-treated Cd- or turpentine-injected ADREX rats were very similar to those in the respective intact animals. Microscopically, the tissues of control rats 24 h after subcutaneous injection of saline were essentially normal.

DISCUSSION

The experimental design used in the present work allowed us to study four distinct levels of adrenal function and/or responsiveness in the altered zinc homeostasis which accompanies inflammation, i.e., in the presence and total absence of hormone availability or responsiveness and after hormone replacement using physiologic and pharmacologic doses.

The findings described strongly indicate that the induction of hepatic metallothionein is not mediated by adrenal hormones in a manner analogous to the glucocorticoid-dependent de novo hepatic synthesis of the acute-phase protein, α_2MFP. In addition, the data presented do not support a

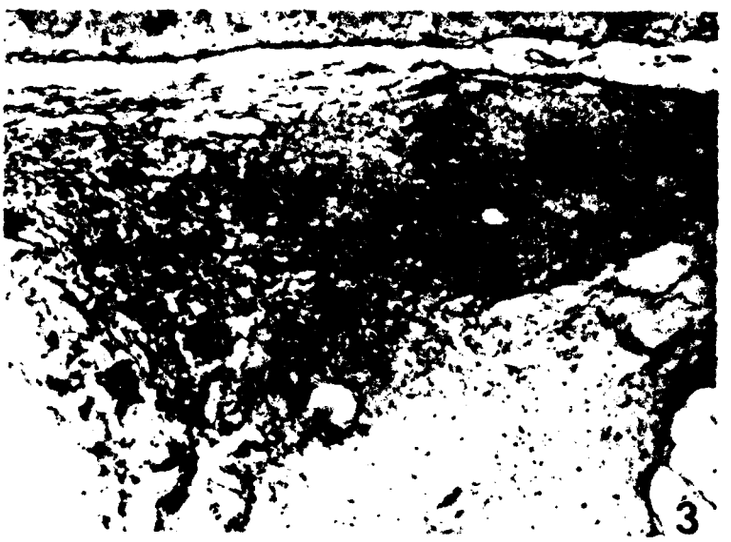

Fig. 3. Cd-treated intact rat. Several dilated and congested vessels, some of which are surrounded by small amounts of hemorrhage within the necrotic and edematous subcutis. ×100 (reduced 10% for reproduction).

major in vivo role of adrenal hormones in mediating the Zn redistribution from plasma to liver which occurs in inflammation. In these studies, adrenalectomy was complete as evidenced by the failure of ADREX rats to manifest an α_2MFP response.

Our results confirm earlier work that showed that inflammation, whether induced by the administration of a heavy metal or other diverse substances (5), occurs concomitantly with hepatic metallothionein accumulation and hypozincemia. Although leukocytic endogenous mediator (LEM), a crude preparation obtained from stimulated polymorphonuclear leukocytes, has been suggested as the endogenous mediator for inflammation-induced redistribution of Zn from plasma to liver as well as for acute-phase protein synthesis (19), no direct effect of this substance (at a dose of 5 μl) on Zn accumulation in isolated hepatocytes has been demonstrated (11). However, administration of approximately 1 ml of crude LEM to rats does induce hypozincemia and hepatic accumulation of metallothionein-like proteins (20) and hepatic synthesis of α_2MFP (21). Thus, it appears likely that LEM may indirectly, rather than directly, affect Zn homeostasis. The role of LEM as such a mediator thus remains undefined.

Fig. 4. Cd-treated intact rat. Aggregates of polymorphonuclear cells associated with fragments of necrotic muscle fibers of the cutaneous trunci. Fewer numbers of inflammatory cells infiltrate the adjacent necrotic and edematous subcutis. ×100 (reduced 10% for reproduction).

In the present studies, the amount of metallothionein found in livers of Cd-treated rats greatly exceeded that measured after the administration of turpentine. Thus, Cd appears to be a more potent stimulus for hepatic metallothionein accumulation when compared to turpentine. However, it is well known that the half-life of Cd–thionein exceeds that of Zn–thionein (22); hepatic accumulation of this metalloprotein reflects the net result of hepatic rates of synthesis and degradation. It is therefore possible that the differences observed in metallothionein content can be attributed in part to differences in turnover rates. On the other hand, both substances produced severe inflammatory responses. Although the nature of the inflammatory lesions produced by these substances is quite different with respect to the extent of necrosis and polymorphonuclear leukocyte infiltration, it is unclear whether these differences contribute significantly to the observed alterations in Zn homeostasis. In other work from this laboratory (5), the severity of gastrointestinal lesions produced by indomethacin, over a limited dose range, was correlated with the extent of hypozincemia and hepatic metallothionein accumulation.

The results presented provide strong evidence for the first time that the hepatic synthesis of metallothionein in inflammatory stress is not mediated to a significant degree in vivo by adrenal hormones. Further, results suggest that mechanisms involved in the in vivo induction of metallothionein synthesis after the administration of heavy metal salts and other diverse agents may be more complex than previously assumed, in that the potential contribution of endogenous Zn redistribution induced by an inflammatory response should be considered. To date, numerous non-heavy-metal substances have been shown by us and others to induce hepatic metallothionein synthesis when injected into rats (5, 6, 23). Most recently, Kotsonis and Klaassen (23) reported an increase in hepatic metallothionein concentration after the administration to rats of sodium iodoacetate, bromobenzene, diethylmaleate, and iodomethane. Although these substances used by them share a common property with regard to their potential to alter sulfhydryl groups, we question whether these agents induce metallothionein accumulation because of this common property as suggested by the authors, or because of their particular irritant properties.

It seems apparent that inflammatory stress constitutes a major factor which influences the synthesis of metallothionein and that its induction after the administration of certain phlogistic agents is independent of adrenal cortical control. Further, our findings support the concept that an increase in intracellular Zn concentration is sufficient for the induction of metallothionein synthesis through metal-stimulated mechanisms involving transcriptional and translational processes (7, 8, 24). Data presented by Failla and Cousins (11) indicate the high concentrations of zinc (98 μM) in dexamethasone-free culture medium is sufficient to promote metallothionein synthesis in isolated rat liver cells. Other evidence to support the important role of Zn in metallothionein induction during stress has been provided by Oh et al. (6). These workers found that "essentially no induction of liver MT [metallothionein] occurred after CCl_4 injection in Zn-deficient rats," whereas CCl_4 stimulated a 543% increase in hepatic metallothionein-Zn in normal animals compared to controls. Our findings also support the possibility that potent glucocorticoids, such as dexamethasone, can influence Zn homeostasis and metallothionein synthesis, albeit to a limited degree, though "primary inducer" mechanisms as suggested by Karin et al. (9). The physiological importance of the role of glucocorticoids in altered Zn homeostasis which accompanies other stress conditions, such as cold environment and strenuous exercise (6), remains to be determined.

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